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Leukemia-Associated RhoGEF (LARG) is not critical for RhoA regulation, yet is important for platelet activation and thrombosis in mice

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Summary. *Background:* RhoA is an important regulator of platelet responses downstream of $G\alpha_{13}$, yet we still know little about its regulation in platelets. Leukemia-Associated RhoGEF (LARG), a RhoA GEF, is highly expressed in platelets and may constitute a major upstream activator of RhoA. To this end, it is important to determine the role of LARG in platelet function and thrombosis. *Methods and Results:* Using a platelet-specific gene knockout, we show that absence of LARG results in a marked reduction in aggregation and dense-granule secretion in response to the thromboxane mimetic, U46619, and PAR4-activating peptide, AYPGKF, but not to ADP. In a ferric chloride thrombosis model *in vivo*, this translated into a defect, under mild injury conditions. Importantly, agonist-induced RhoA activation was not affected by absence of LARG, although basal activity was reduced, suggesting that LARG may play a house-keeper role in regulating constitutive RhoA activity. *Conclusions:* LARG plays an important role in platelet function and thrombosis *in vivo*. However, although LARG may have a role in regulating the resting activation state of RhoA, its role in regulating platelet function may principally be through RhoA-independent pathways, possibly through other Rho-family members.

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Key words:

Larg protein, mouse; platelets; thrombosis; gene knockout; RhoA protein, mouse

Introduction

Platelets are critical for the formation of thrombi in blood vessels leading to rapid occlusion and tissue infarction. An understanding of their function, at a molecular level, is vital to enable a better understanding of the pathophysiology of thrombotic ischemic disease and development of approaches for diagnosis and management. Platelets express multiple members of the Rho family of small GTPases, whose role in many cell types is associated with actin cytoskeletal rearrangements and membrane and granule trafficking (1-4). In turn these GTPases are regulated by the balanced activities of Rho activators, the guanine nucleotide exchange factors (GEFs), and Rho inhibitors, the GTPase activating proteins (GAPs). Oligophrenin-1 has recently been shown to be an important RhoGAP in platelets (5), but the activity of expressed RhoGEFs in platelets are currently sparsely reported.

The most abundantly expressed RhoGEF in platelets is Leukemia-Associated RhoGEF (LARG, also known as ARHGEF12) (6), a G-protein coupled receptor (GPCR)-regulated GEF. Previously, RhoA has been described as an important regulator of platelet function and development, with the generation of a platelet-specific RhoA knockout showing macrothrombocytopenia, reduced platelet function in response to the thromboxane mimetic, U46619, and thrombin, and also reduced thrombus formation *in vivo* (4). Based on the high level of LARG expression in platelets, and its specificity for RhoA (7), we hypothesized that LARG would be the principal signal transducer from $G\alpha_{13}$ -coupled receptors to RhoA (8).

Here we present data that demonstrates LARG is an important regulator of platelet function and thrombosis *in vivo*, but suggests agonist-induced RhoA activation does not primarily depend on LARG. As such, the regulation of RhoA may principally be mediated by GEFs other than LARG, and that LARG may act independently of RhoA.

Methods

Mice

Mice carrying a floxed allele of the gene encoding LARG (*Arhgef12*) were generated as previously described (9) and crossed with PF4-Cre positive mice for generation of platelet-specific knock-outs. Studies were approved by the local research ethics committee at the University of Bristol, UK, with mice bred and maintained under the UK Home Office project license PPL 30/2908.

Preparation of PRP

PRP was prepared from heparinized blood (100U/mL 1:10 v/v) taken via cardiac puncture and supplemented with 10U/mL heparin (1:5 v/v). Briefly, heparinized blood was spun in a fixed bucket microcentrifuge at 200x *g* for 5 mins. The PRP and top third of the erythrocyte layer were spun again in a swing bucket centrifuge at 200x *g* for 6 mins. PRP was standardized to 2×10^8 plts/mL in modified HEPES-Tyrode's buffer. The residual erythrocyte layer was spun at 1000x *g* in a fixed bucket microcentrifuge. PPP was removed and diluted to the same extent as the appropriate PRP.

Washed platelet preparation

Washed platelets were prepared from citrated blood (4% sodium citrate, 1:10 v/v) taken via cardiac puncture and supplemented with ACD (1:7 v/v). Platelets were pelleted from PRP in the presence of 140 nM prostaglandin E₁ and 0.02 units/mL apyrase. The platelet pellet was resuspended at a concentration of 2×10^8 plts/mL in modified HEPES-Tyrode's buffer containing 0.02 units/mL apyrase. For certain assays, 10 μ M indomethacin was added before and after the pelleting stage.

Western blotting

Western blotting samples were prepared from washed platelet preparations. Rabbit anti-LARG (clone H70) and rabbit anti-RhoA (clone 26C4) were from Insight Biotechnology (Wembley, UK). Mouse anti- α -tubulin was from Sigma UK (Poole, UK).

Flow cytometry

Washed platelets at 2×10^7 plts/mL were incubated with 1/25 FITC-conjugated rat anti-mouse antibodies against CD41/ α_{IIb} , GPVI and GPIIb α , or isotype-specific controls, for 10 minutes at room temperature, prior to fixation with an equal volume of 4% paraformaldehyde. Samples were analysed on a BD FACS Canto II flow cytometer with FACS Diva software.

Lumiaggregometry

Lumiaggregometry was performed on a ChronoLog Corp. Model 560-VS aggregometer and Aggrolink 5. software. Manufacturer's instructions were followed, with the exception that the aggregations were scaled down to 250 μ L. Maximum extent and rate of aggregation were calculated using the Aggrolink 5 software.

Thromboxane B₂ ELISA

Releasates were generated under aggregating conditions before being quenched with 5 mM EDTA and 200 μ M indomethacin. Releasates were centrifuged for 3 minutes at 10,000x *g*, decanted and snap frozen. Releasates were diluted 1/50 prior to analysis of thromboxane B₂ levels via a commercial ELISA kit (Enzo Life Sciences, Exeter, UK) according to the manufacturer's instructions as previously described (10).

RhoA G-LISA

Washed platelets were stimulated under non-stirring conditions before the addition of an equal volume of ice-cold 2x lysis buffer (100 mM Tris-Cl, 1 M NaCl, 20 mM MgCl₂, 2% Triton X-100) containing Complete, EDTA-free protease inhibitors (Roche Diagnostics Ltd, Burgess Hill, UK). Lysates were analysed via commercial RhoA G-LISA kit (Cytoskeleton Inc., Universal Biologicals, Cambridge, UK) according to the manufacturer's instructions.

Spreading assays

Platelets containing indomethacin at 4×10^7 plts/mL were added onto coverslips coated in 50 µg/mL collagen-related peptide (CRP) or 100 µg/mL fibrinogen, or BSA as a control, for 30 minutes at room temperature. Cells were fixed with an equal volume of 8% paraformaldehyde, washed twice with PBS and stained with 2 µM DiOC₆ for 1 hour at room temperature. Platelets were washed twice before being mounted with MOWIOL solution containing DABCO. Platelets were imaged on a Leica DM IRM epifluorescence microscope with 5 random fields of view captured using Volocity Image Analysis software (Perkin Elmer, Coventry, UK). Images were analysed using ImageJ 1.46

***In vivo* thrombosis assays**

Mice were anesthetized with 100 mg/kg ketamine (Vetalar V, Pfizer) and 10 mg/kg xylazine (Rompun, Bayer). Platelets were labelled by intravenous administration of 100 mg/kg Dylight 488-conjugated anti-GPIIb/IIIa antibody (Emfret Analytics, Eibelstadt, Germany). Right arteries were exposed and 2 x 1 mm pads of filter paper soaked with ferric chloride in PBS were applied for 3 minutes. Injury sites were imaged by time-lapse microscopy for 20 minutes and images were analysed by ImageJ 1.46. Background fluorescence values measured upstream of the injury site were subtracted from the thrombus-specific fluorescence. Data are expressed as integrated fluorescence density.

***In vitro* thrombosis assays**

In vitro thrombosis assays were performed on a parallel-plate flow chamber. Coverslips were coated with 50 µg/mL HORM collagen for 2 hours at room temperature before being blocked overnight at 4°C with 1% fatty acid free BSA (Sigma, Poole, UK). Assembled chambers were blocked for 30 minutes at room temperature prior to a run. Blood was taken into 4% citrate (1/10 v/v) supplemented with 2 units/mL heparin and 40 µM PPACK. Blood was incubated with 2 µM DiOC₆ for 10 minutes at room temperature. Blood was flowed at 1000 s⁻¹ for 2 minutes. Images were captured by time-lapse microscopy, with 5 randomly selected fields of view captured for end-point analysis. Images were analysed by ImageJ 1.46.

Results and Discussion

Platelets from LARG:PF4-Cre⁺ (knockout, KO) mice were confirmed to be deficient in LARG, yet still expressed wild-type levels of RhoA (Figure 1A). Hematologic analysis of knockout mice showed that absence of LARG from platelets did not impair hematopoiesis. Likewise, the absence of LARG in

platelets did not affect the basal cell surface expression of key platelet glycoproteins α_{IIb} , GPIb and GPVI (Table 1). This is unlike the RhoA knockout where a macrothrombocytopenia was present (4), indicating that the absence of LARG, unlike RhoA, does not impact the development and generation of platelets *in vivo*.

Aggregation of knockout platelets in PRP was diminished in response to the thromboxane mimetic, U46619, and the PAR4 receptor-stimulating peptide (PAR4-P), AYPGKF, but not to ADP. Dense granule secretion, as measured by ATP release in a luciferin-luciferase assay (Figure 1B) was also reduced in knockout platelets; however, thromboxane generation was not (Figure 1C). The defect in thromboxane and PAR4-dependent responses and not those to ADP suggests that LARG is important in $G\alpha_{12/13}$ -linked GPCR responses, and not those linked to $G\alpha_i/G\alpha_q$. Adhesion and spreading of LARG-deficient platelets on fibrinogen or collagen-related peptide (CRP) under static conditions were also normal (Figure 1E), suggesting that LARG does not act downstream of $\alpha_{IIb}\beta_3$ integrin-associated $G\alpha_{13}$ (11).

Importantly, RhoA activity was reduced in knockout platelets compared to wild-types in both resting and agonist-stimulated platelets, when measured by G-LISA (Figure 1D). When normalized to basal activity (not shown), there was no longer a difference in stimulated RhoA activity between controls and LARG-deficient platelets, suggesting that LARG regulates basal RhoA activity, but is not critical for activating RhoA after platelet stimulation. LARG was a good candidate to be the primary GEF regulating RhoA activity in platelets, so it is therefore surprising that RhoA activity was not altered in an agonist-dependent manner. It could be that LARG plays a “house-keeping” role maintaining a background level of RhoA activation, such that its absence results in a cell that is less responsive, and that RhoA agonist-dependent activation is through other RhoGEFs. The uncoupling of the apparent functional roles of LARG from RhoA activation may also be due to LARG regulating other Rho-family member in platelets (12). However, given that both RhoF (2) and RhoG (1, 13) knockout mice present dissimilar phenotypes, these Rho-family members are unlikely to be regulated by LARG, suggesting that RhoB or RhoC may be involved, despite their low copy numbers (14). It has also been reported that LARG can form homodimers or heterodimers with PDZ-RhoGEF, via interactions with their inhibitory C-terminal domains that may recruit other inhibitory factors (15). Although PDZ-RhoGEF is not reported to be expressed in platelets at the protein level (14), the suggestion that dimerization may recruit other inhibitory factors could be important for explaining the regulatory role of LARG in platelets.

Analysis of thrombus formation *in vivo* revealed knockout mice were also protected from thrombosis induced at lower (6%) concentrations of $FeCl_3$ (Figure 2A), although at higher concentrations (12%) of $FeCl_3$, this protection was overcome (Figure 2B). Likewise, thrombus formation on collagen-coated surface *in vitro*, under non-coagulating conditions, showed no difference between wild-type and knockout mice (Figure 2C). The presence of an *in vivo* defect under milder injury conditions is consistent with a defect in thrombin-dependent thrombus formation (16). The absence of a difference in thrombus formation under non-coagulating conditions *in vitro*, where thrombin is not generated, is also consistent with a $G\alpha_{12/13}$ -link.

We conclude that the Rho-GEF LARG has an important role in regulating platelet aggregation downstream of $G\alpha_{13}$ -coupled receptors (thrombin and thromboxane receptors). Importantly this translates into a defect in arterial thrombosis *in vivo*. Although we had hypothesised that LARG may be operating through RhoA to mediate these effects, the difference in phenotypes between LARG-deficient and RhoA-deficient platelets, and our discovery here that only basal RhoA activity is affected by absence of LARG, points to other pathways that LARG may regulate to mediate its effects. Given the marked effect upon dense granule secretion, we suggest that LARG may be operating as a novel regulator of secretion downstream of $G\alpha_{13}$ -coupled receptors.

Author contributions

C. M. Williams designed and performed experiments, analysed data and wrote the manuscript. M. T. Harper, R. Goggs and T. G. Walsh designed and performed experiments, analysed data and edited the manuscript. S. Offermanns provided essential reagents and edited the manuscript. A. W. Poole designed experiments and wrote the manuscript.

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Disclosures

The authors state that they have no conflict of interest.

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Tables

Table 1 Hematology and platelet surface receptor analysis

		WT			KO		
		Mean	SEM	N	Mean	SEM	N
Hematology	WBC* ($10^3/\text{mm}^3$)	7.26	0.70	11	5.73	0.70	11
	RBC† ($10^6/\text{mm}^3$)	10.13	0.19	11	10.18	0.24	11
	PLT‡ ($10^3/\text{mm}^3$)	749.6	47.2	11	804.0	56.9	11
	MPV§ (μm^3)	5.32	0.15	11	5.26	0.06	11
Surface receptors	α_{IIb}	3127	115	4	3139	212	5
	GPIb	713.8	53.6	4	754.5	49.2	5
	GPVI	320.9	9.7	4	325.8	11.7	5

Hematology data of citrated whole blood was acquired using a Horiba Pentra ES60. Platelet surface receptors were determined by flow cytometry. Washed platelets from WT and KO mice were incubated with fluorescently-conjugated antibodies against α_{IIb} , GPIb and GPVI then fixed. Data are presented as geometric means. *White blood cells. †red blood cells. ‡platelets. §mean platelet volume.

Legends

Figure 1 LARG null platelets are developmentally normal, but demonstrate reduced activity to the thromboxane mimetic U46619 and PAR4-peptide AYPGKF

(A) Platelet lysates from WT and KO mice were probed via western blotting (n=3). (B) PRP from WT and KO mice was analysed via lumiaggregometry to determine (i) the maximum extent of aggregation within 3 minutes, (ii) the maximum rate of aggregation and (iii) the amount of ATP secretion as a readout of dense-granule secretion (n≥3). Data were compared by 2-way ANOVA with *** = $P < 0.001$. (C) PRP from WT and KO mice was stimulated under stirring conditions for 5 mins. Reactions were stopped with 5mM EDTA and 200 $\mu\text{mol/L}$ indomethacin. Releasates were harvested via centrifugation before being analysed for TXB_2 levels by ELISA (n≥3). (D) RhoA activity was measured by G-LISA. Washed platelets from WT and KO mice at 2×10^8 plts/mL, supplemented with 0.02 units/mL apyrase, were stimulated under non-stirred conditions for 5 minutes at 37°C (n=7). Data are shown as optical density (OD) and were compared by 2-way ANOVA with * = $P < 0.05$, ** = $P < 0.01$ and *** = $P < 0.001$. (E) Washed platelets were applied to BSA, CRP or fibrinogen-coated surfaces for 1 hour prior to fixing and staining. Adhesion was determined as being the number of adhered platelets per field of view (i) and platelet spread area was determined by fluorescence (ii). Platelets applied to fibrinogen-coated surfaces were co-stimulated with either 0.1 units/mL or 1.0 unit/mL thrombin to stimulate spreading on that surface.

Figure 2 Mice with LARG null platelets demonstrate reduced thrombus formation in response to low level injury in vivo

Exposed carotid arteries from WT and KO mice were injured with (A) 6% (WT n=10, KO n=11) or (B) 12% ferric chloride (WT n=8, KO n=6) for 3 minutes. Thrombus formation was monitored for 20 minutes. Data were analysed using ImageJ 1.46 yielding (i) the mean integrated fluorescence density over time, (ii) the thrombus size after 20 minutes. Representative images of thrombi from (iii) WT and (iv) KO. Data were compared by unpaired *t*-test where appropriate. * = *P*<0.05. (C) Whole blood was flowed through a parallel-plate flow chamber over a collagen-coated surface. Platelet accumulation was followed for 2 minutes by time-lapse microscopy (i) with stills taken for end point analysis (ii). Representative images of thrombi from (iii) WT and (iv) KO.

Figure 1

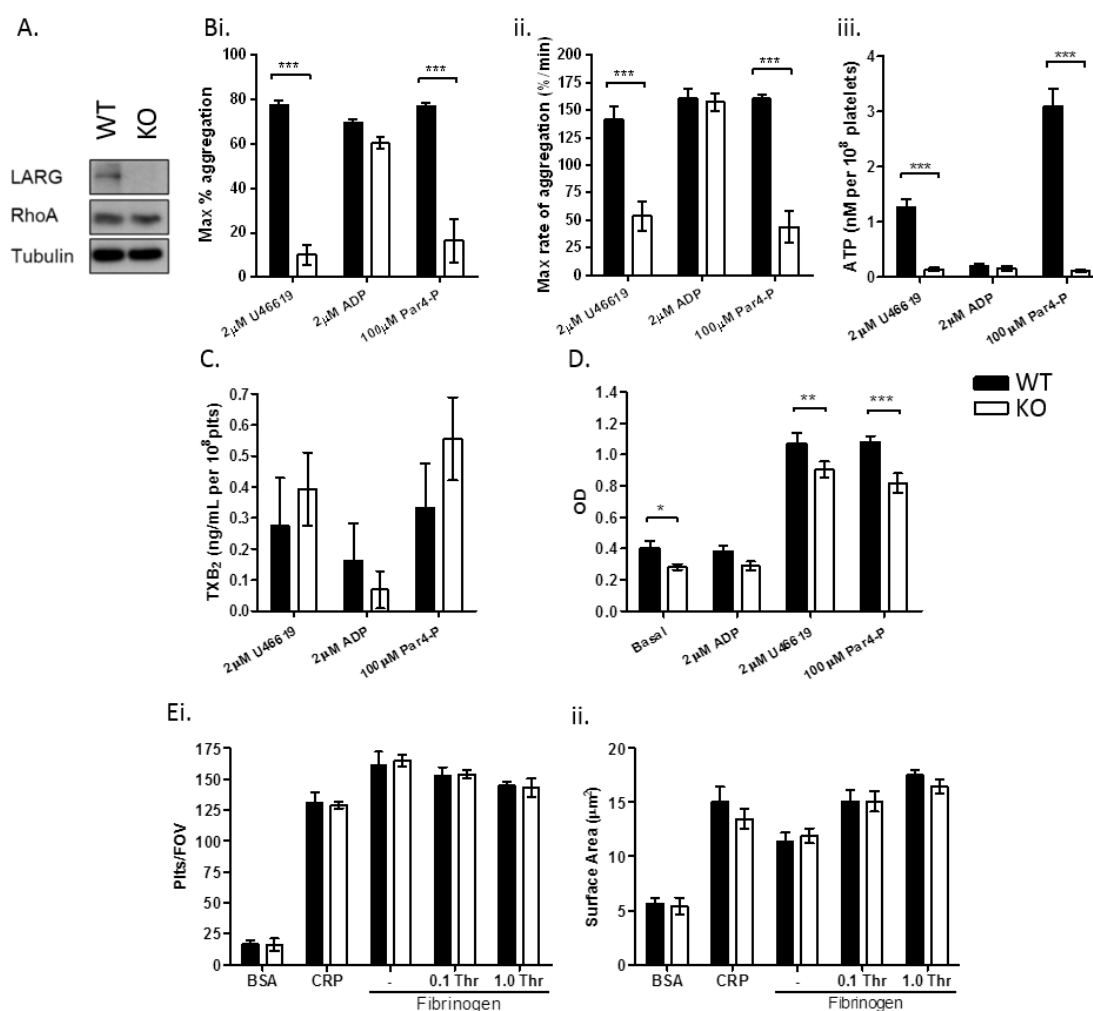


Figure 2

